Supporting Information

Repurposing low–molecular-weight drugs against the main protease of severe acute respiratory syndrome coronavirus 2

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Experimental Procedures

Protein expression and purification

DNA encoding $M^{pro}$of SARS-CoV-2 (residues 1-306) was amplified and incorporated into a modified pGEX-4T-1 (Novagen) plasmid, in which the thrombin protease site was replaced by the tobacco etch virus (TEV) cleavage site. *Escherichia coli* BL21(DE3)-RIL cells (Novagen) were cultured in LB mediumat 37℃ till OD_{600nm} reached0.8-1.2, and induced by 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 16 ℃ for 24 hours. Bacterial pellet was resuspended in buffer A (20 mM Tris, 1 M NaCl, 2 mM DTT, 1 mM EDTA at pH 7.6) and lysed by sonication on ice. The GST-tagged protein was purified on glutathione-Sepharose beads (GE Healthcare), then treated with TEV to cleave the N-terminal GST tag overnight at 16℃. It was then purified usinga Superdex 200 column (GE Healthcare). Finally, the purified full-length M^{pro} protein (residues 1-306) was concentrated to about 10 mg/ml in buffer B (20 mM Tris, 150 mM NaCl, 2 mM DTT, 1 mM EDTAat pH 7.6) and stored at -80 ℃ for ligand-observed NMR screening, crystallographic study andenzymatic assay.

The N-terminal (residues 4-199) and C-terminal (residues 187-306)$M^{pro}$of SARS-CoV-2were used for protein-observed NMR studies.DNA encoding N-terminal M^{pro} proteins and C-terminal M^{pro} were cloned into the pET22b vector (GE Healthcare). The reconstruct plasmids were transformed to Escherichia coli BL21(DE3)-RIL. The bacteria cells were cultured in the minimal medium supplemented with $^{15}NH4Cl$, and induced at OD600nm of 0.8-1.2 by 0.4mM IPTG at 16 ℃ for 24 hours. The proteins were further purified using Ni-chelating column (Qiagen, GE Healthcare) and gel filtration chromatopraphy (Superdex-200).The purifedN-terminal and C-terminal Mproproteins were concentrated to a buffer containing 20mM Bis-

Tris,150mM NaCl, and 2mM DTT at pH 5.9.

NMR spectroscopy

All NMR spectra were acquired at the Agilent 700MHz spectrometer equipped with a cryoprobe. The ligand-based NMR spectra, i.e., 1D ^{1}H incorported with WATERGATE for solvent suppression,the saturation transfer difference (STD),WaterLOGSY and CPMG spectra, were acquired to identify weak binders. The 1D ^{1}H spectrum was acquired with2 s acquisition and 2 s relaxation delay per scan and 32 number of transients. The STD spectrum was acquired within 15 minutes approximately using 1 s acquisition time,32 dummy scans, 0.1 s relaxation delay, followed by a 2 s Gauss pulse train with the irradiation frequency at -0.7 ppm or -50 ppm alternatively, and 256 scans. WaterLOGSY was acquired for 15.1 minutes with 1 s acquisition time, 1 s relaxation and 1.3 s NOE mixing time and 256 scans.The T2 relaxation time of 0.8 s was applied in the Carr-Purcell-Maiboom-Gill (CPMG) spectrum, while other parameters remained similar to the 1D ^{1}H spectrum.

The NMR HSQC titration was performed to probe chemical shift perturbations of the ^{15}N-labeled N- or C-terminal M^{pro} of SARS-CoV-2 induced by small molecules. The HSQC spectra were acquired using 1.3 srelaxation delay, 32increments in the ^{15}N dimension and 32-64 scans per increment at 308 K. The HSQC spectra of M^{pro}-Cof SARS-CoV-2 were collected at 298K.The compounds were stocked atDMSO-d_6 at a concentration of 20 mM.The 0.1mM ^{15}N-labeled $M^{pro}$was titrated at the ligand/protein molar ratio ranging from 0.0 to 8.0.

Enzymatic assay of SARS-CoV-2 M^{pro}

To characterize the enzymatic activity of SARS-CoV-2 M^{pro} and to measure the inhibition activities of different inhibitors, the fluorescence resonance energy transfer (FRET) based assay

was carried out as described previously(Yang H, Xie W, Xue X, Yang K, Ma J, et al. *PLoS Biol.***2005**, 3: e324). In brief, the enzymatic activity was determined by continuous kinetic assays using the fluorescently labeled substrate, FITC-AVLQSGFR-Lys(Dnp)-Lys-NH2 (over 95% purity, GL Biochem Shanghai Ltd). The fluorescence intensity was monitored with a CLARIO star microplate reader (BMG Labtech) using exciting and emitting wavelengths at 485nm and 520 nm, respectively. The experiments were performed with abuffer consisting of 50 mM Tris-HCl, 1 mM EDTA, 5% DMSO at pH 7.3.The SARS-CoV-2 M^{pro}at a final concentration of 0.5 μM~5 μM was mixed with the inhibitors and incubated at 30℃ for 10 minutes. After centrifuge at 13000 rpm and 4 ℃ for 10 minutes, the supernatant of the mixture was added into the fluorescent-labeled substrate at a final concentration of 10μM~16μM. The fluroresent intensitied were measured immediately,with 20 or 30 seconds interval and a total acquisition time of about 50 minutes. The data was analyzed using Origin 2020 pro (OriginLab Corporation,).

Molecular docking

All structures of chemical compounds were obtained from the DrugBank database. The 3D structures of the small molecules were minimized with Tripos force field after adding Gasteiger and Marsili charges. Crystal structure of M^{pro} (PDB ID : 6LU7) was obtained from PDB database and prepared with the following instructions: All hydrogen atoms were added with H-bonding orientation; All ligands and water were removed; repaired related missing sidechains were repaired and atom bumps were removed with 100 cycles energy minimized. Small molecules were docked with M^{pro} using Autodock Vina software. Docking site was defined as the cubic grid box with side length of 22 angstroms or above centered on the catalytic active site. For virtual screening, the binding modes with the lowest binding energy were recommended for

each molecule.Binding modes of small molecules with M^{pro} were graphed by VMD software (Humphrey, W., Dalke, A. and Schulten, K., *J. Molec. Graphics,* **1996**, 14:33-38).

Tabel **S**1. Fragments as pharmacophores of repurposing drugs proposed from a virtual screening.

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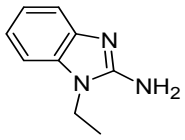
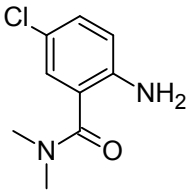
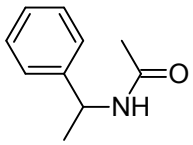
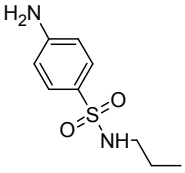
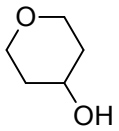
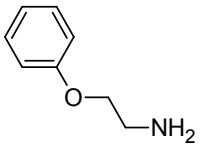
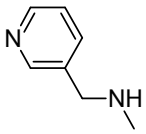
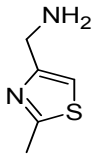
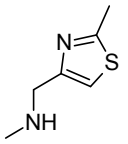
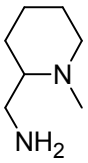
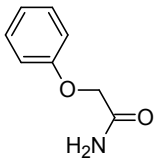
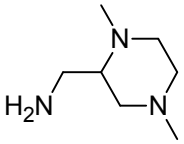
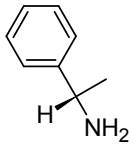
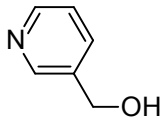
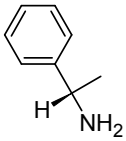
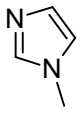
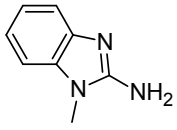
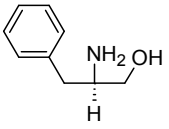
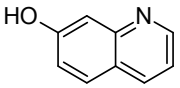
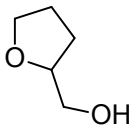
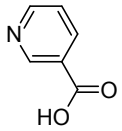
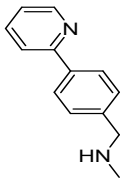
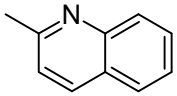
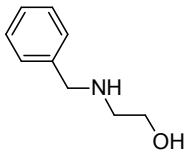
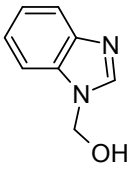
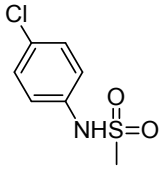
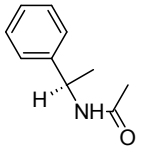
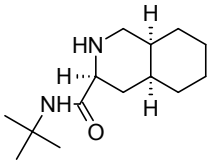
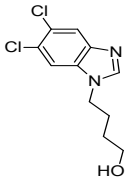
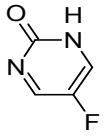
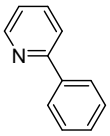
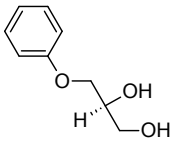
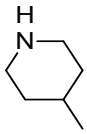
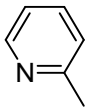
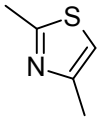
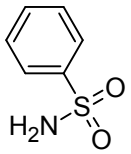
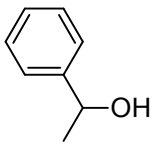
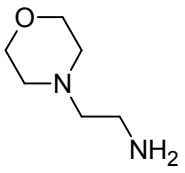
4028271		4034616		5108520	
7993027		CC29915		RF03254	
TL00368		CC05713		CC05746	
MO01154		SPB04334		CC13813	
SB01486		CC04109		SB00810	
AC12699		AC27630		AC41734	

AC41882		AC42044		CC04101	
CC41546		AC13217		AC10594	
XBX00335		KM05166		HTS06398	
SB01998		SPB07625		JFD02014	
JFD03594		MAY00282		AC12751	
AC14933		CD01071		AC14849	
AC35188		AC40075			

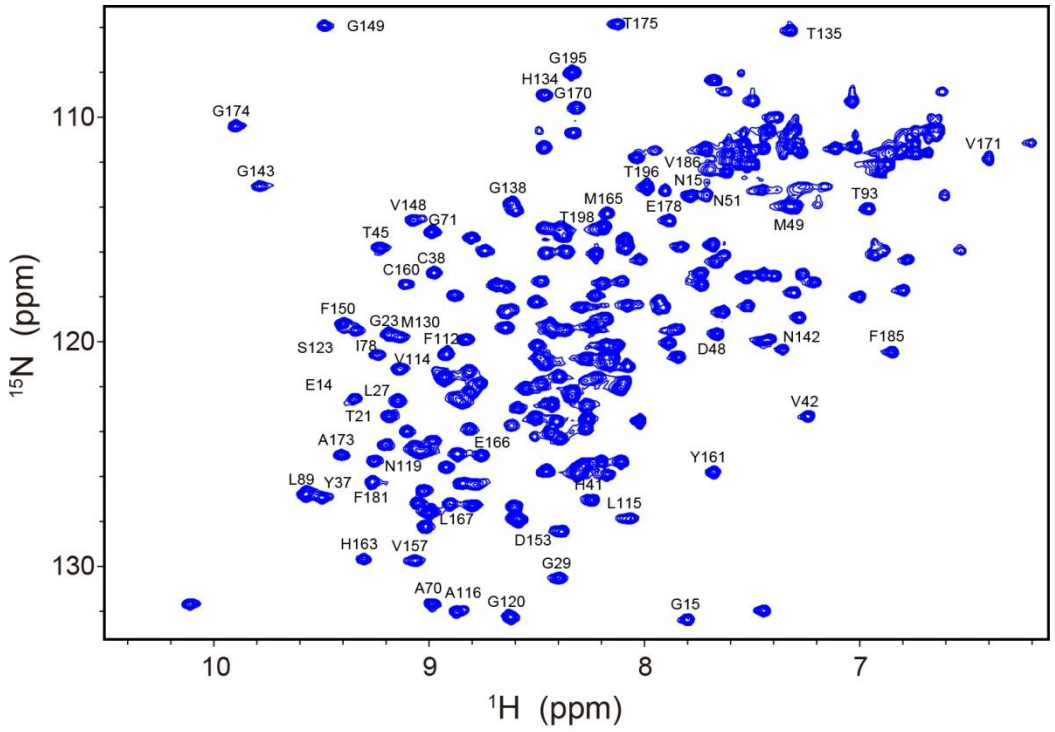


Figure S1. The backbone ^{1}H-^{15}N chemical shift assignment of SARS-CoV-2 M^{pro}-N transferred from its homolog SARS M^{pro}-N.

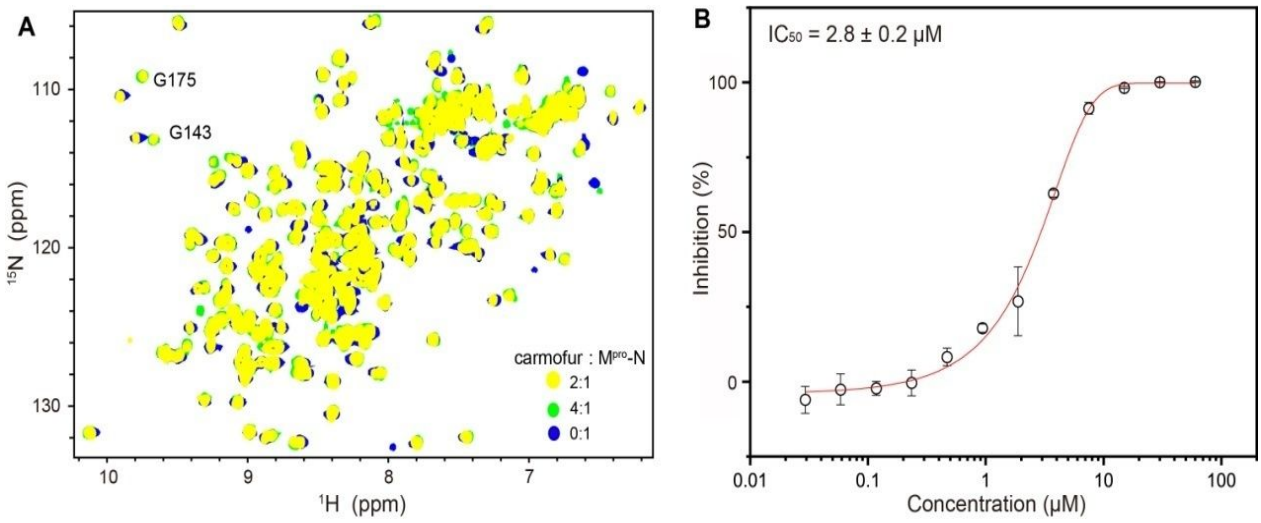


Figure S2. Carmofur binds to the catalytic core and inhibits the enzymatic activity of SARS-CoV-2 M^{pro}. A) Chemical shift changes induced by carmofur at various doses. B) Dose-dependent inhibition profile of carmofur in the presence of 0.5 µM SARS-CoV-2 M^{pro} and 16 µM fluorescence labeled substrate. Errors were estimated from three biological duplicates.

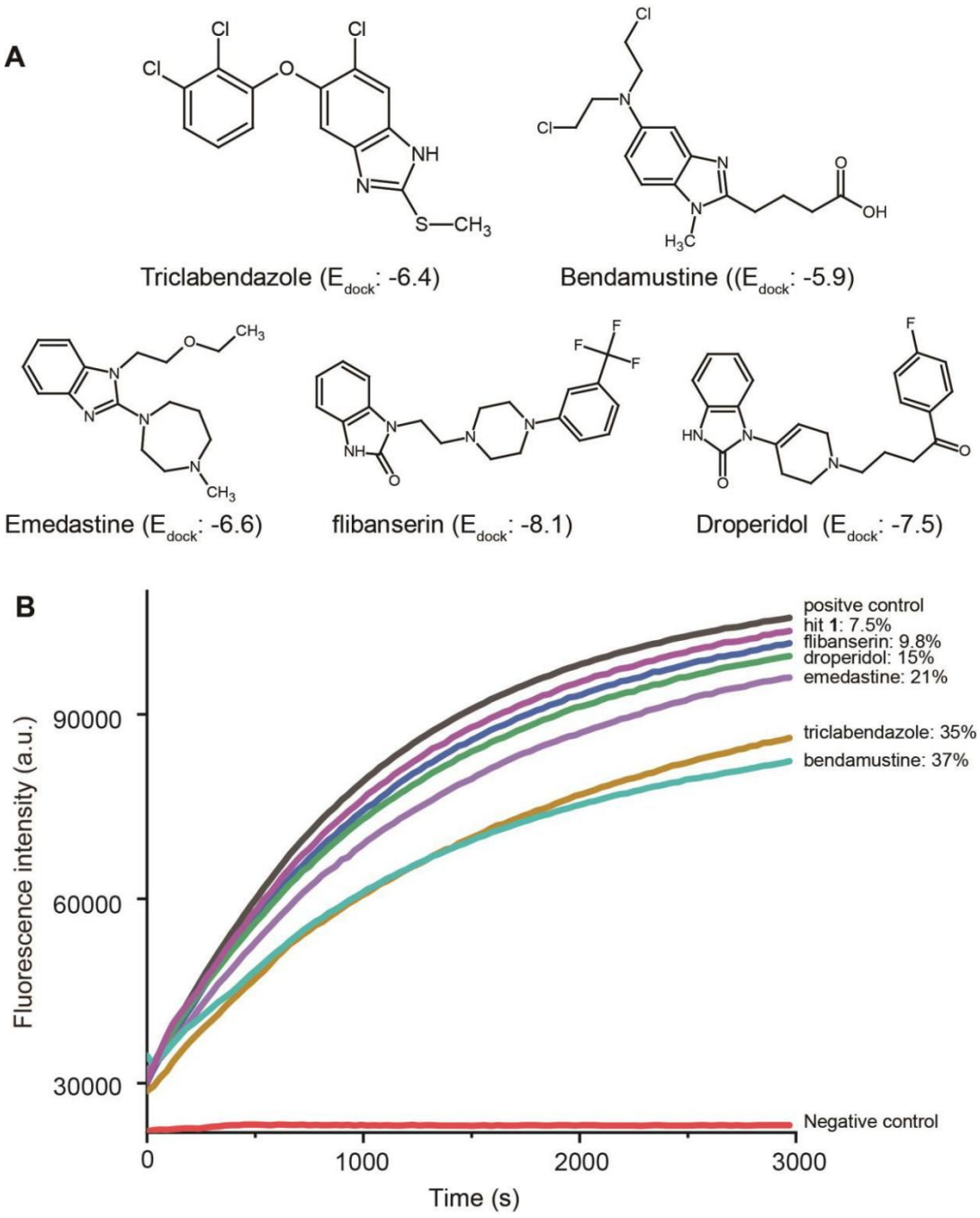


Figure S3. Enzymatic activity of SARS-CoV-2 M^{pro} inhibited by repurposing drugs containing hit **1** pharmacophore. A) The chemical structures of the drugs as hit **1** derivatives with molecular docking energy annotated. B) The single-dose (60 μ M) inhibition of the enzymatic activity of SARS-CoV-2 M^{pro} with inhibition rate annotated.

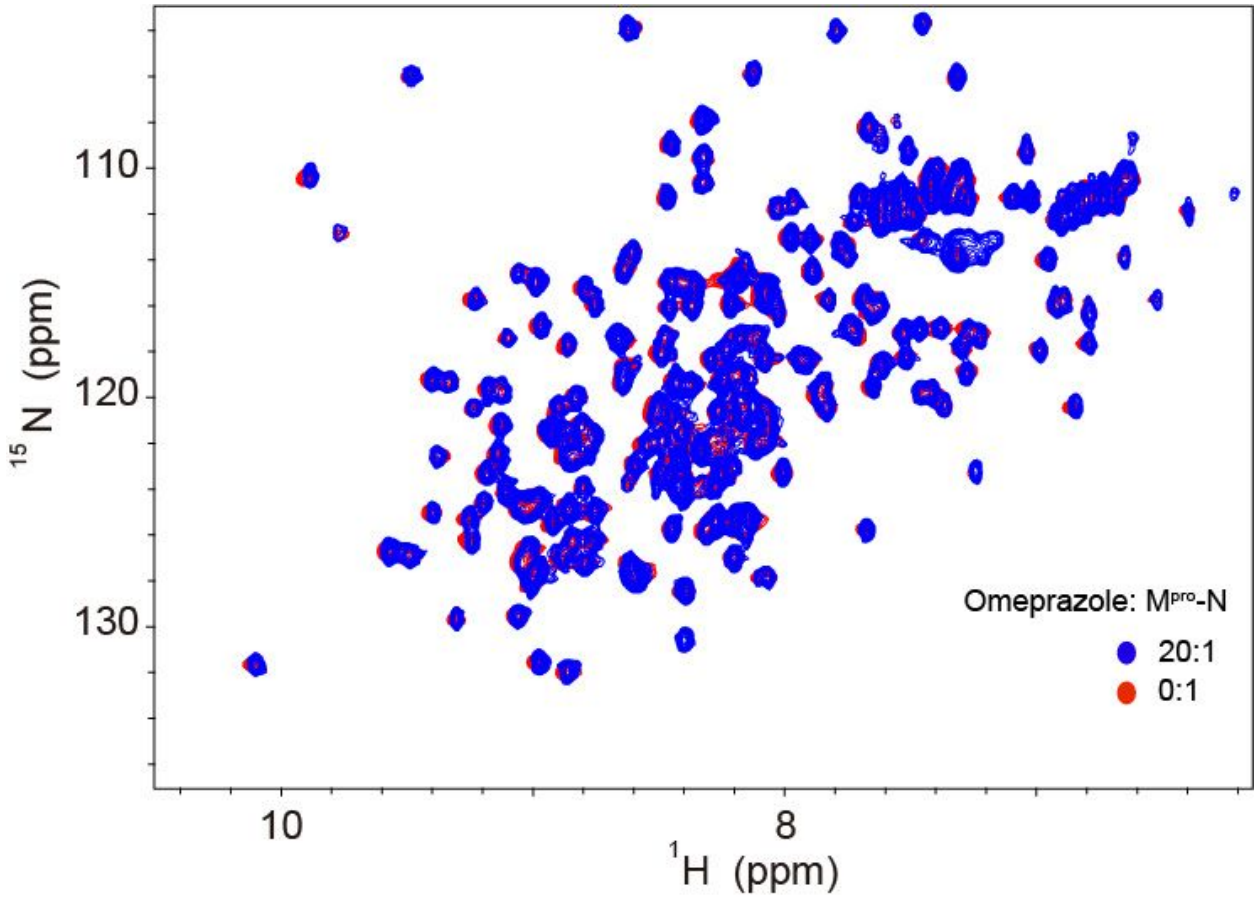


Figure S4. Chemical shift perturbations of ^{15}N-labeled SARS-CoV-2 M^{pro}-N (residues 4-199) induced by omeprazole at the annotated ligand:protein molar ratio.

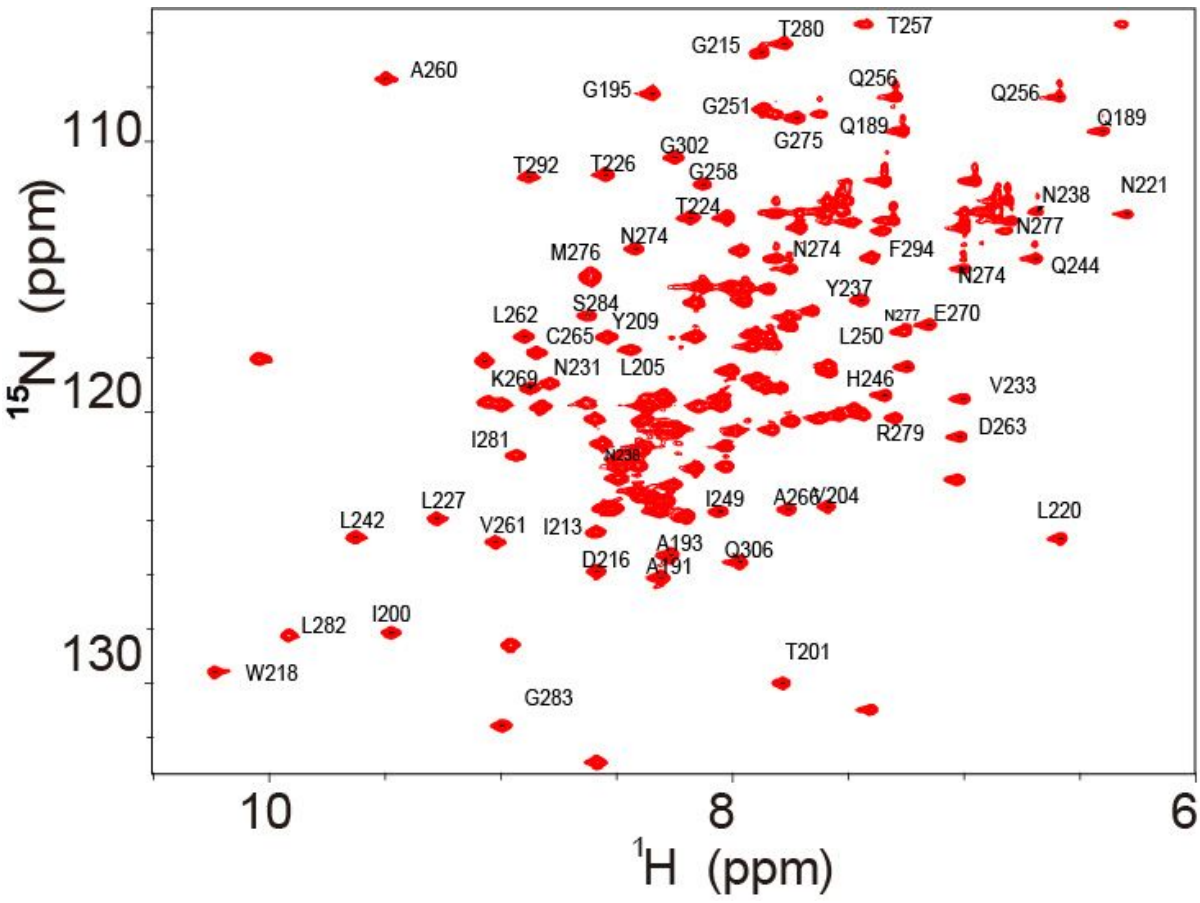


Figure S5. The backbone ^{1}H-^{15}N chemical shift assignment of SARS-CoV-2 M^{pro}-C (residues 187-306) transferred from its homolog SARS M^{pro}-C.

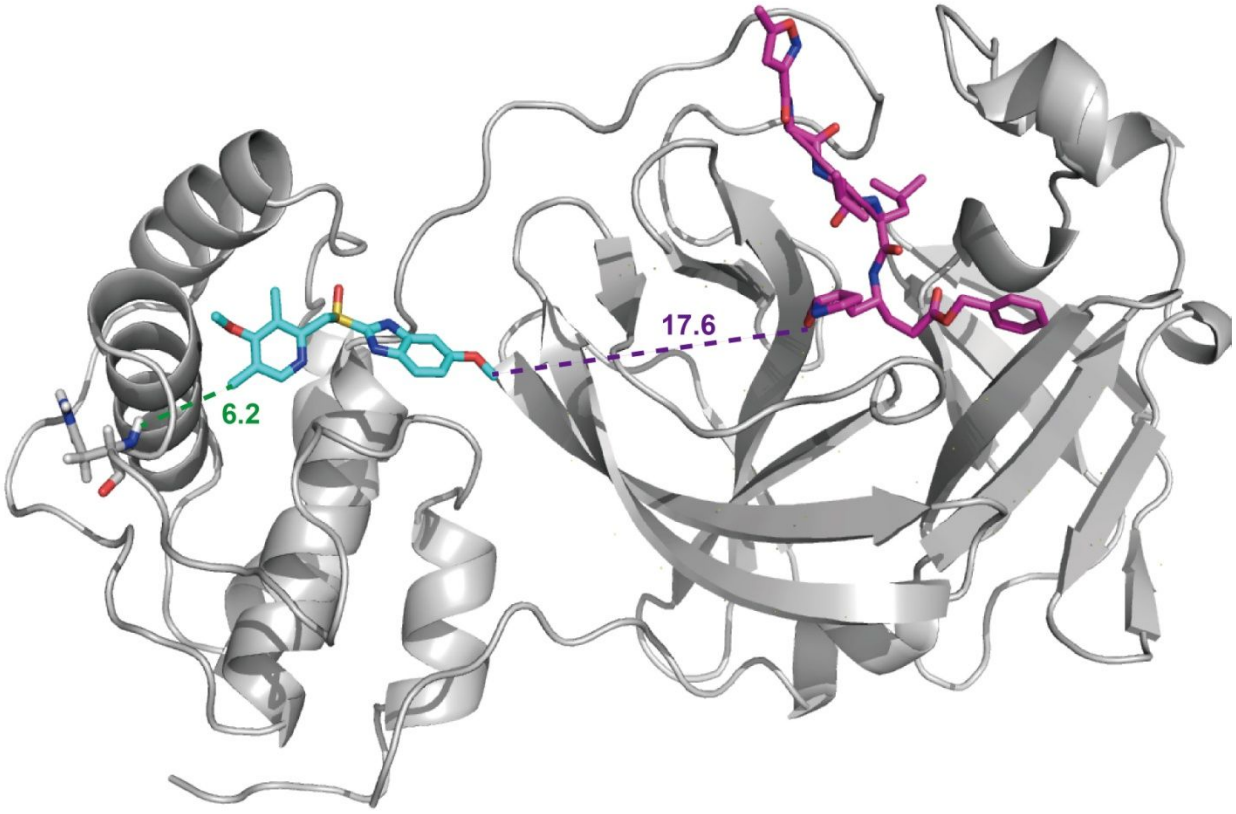


Figure S6. The binding pose omeprazole docked to the C-terminal M^{pro} of SARS-CoV-2 (PDB code:6LU7).Annotated was the nearest distances between omeprazole (carbon atoms colored in cyan) and the N3 inhibitor (magenta) in the N-terminal M^{pro} of SARS-CoV-2, or residue N277 (gray), respectively.

Author Contributions

JG and ZL: experiment and data analysis.XL,FL,RM,ZZ,JZ,JW,YGand YP: resources. YG and KR: conceptualization and writing. JG and ZL contributed equally to this work.

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